

Application No.: 10/506,693
Attorney Docket No.: 47675-86
First Applicant's Name: Kurt Berlin
Application Filing Date: 21 April 2005
Office Action Dated: 06 October 2008
Date of Response: 06 April 2009
Examiner: Katherine D. Salmon

IN THE SPECIFICATION:

Applicants, pursuant to 37 C.F.R. § 1.121, submit the following amendments to the specification:

Kindly *substitute* the following contiguous paragraphs for the corresponding paragraphs beginning on page 43 and extending through page 47 of the originally filed specification:

--EXAMPLE 1

Organ specific methylation pattern analysis on plasma samples

A blood sample ~~is~~was taken from a patient who ~~is~~was unaware that he had been exposed to high levels of radiation during his years of service at the army. Now he wishes to know whether he has developed a neoplastic disease like a tumour. His physician has not yet found any typical symptoms other than the patient complaining about unspecific pain at different organs, including headache.

A 20 ml blood sample ~~is~~was collected in heparin. Plasma and lymphocytes ~~are~~were separated by Ficoll gradient. Control lymphocyte and plasma DNA ~~are~~were purified on Qiagen columns (Qiamp Blood Kit, Qiagen, Basel, Switzerland) according to the "blood and body fluid protocol". Plasma ~~is~~was passed on the same column. After purification of about 10 ml of plasma 350 ng of DNA ~~are~~were obtained. The DNA ~~is~~was subjected to a sodium bisulfite treatment as it has been described in Olek A, Oswald J, Walter J. (1996) A modified and improved method for bisulphite based cytosine methylation analysis. Nucleic Acids Res. 24: 5064-6. An aliquot of this bisulfite treated DNA ~~is~~was used for methylation analysis based on sequencing. The individual's test result ~~is~~was compared with the dataset obtained from previous samples of known tissues and cell types as it is shown in figure 7. From that it ~~can~~could be concluded that a significant portion of the DNA in the patient's blood ~~is~~was derived from his lung. Said result ~~is sent~~was send back to the physician who now ~~refers~~referred the patient to a hospital that is specialised on inflammatory or

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cell proliferative diseases of the lung.

EXAMPLE 2

Organ specific methylation pattern analysis on serum samples

A blood sample ~~is~~was taken from a patient, who wishes to know whether he has developed a neoplastic disease like a tumour. His physician has not yet found any typical symptoms other than the patient complaining about randomly occurring unspecific pain in his stomach, recurrent headache and pain in his kidneys.

A serum sample ~~is~~has been taken from the patient. DNA ~~is~~has been isolated from the serum with the use of the Qiamp kit and ~~is~~has been bisulfite treated as described in Example 1.

A typical methylation pattern ~~can~~could be determined analysing the methylation statuses of a higher number of different informative CpG sites, ~~than is~~that were used as markers for different tissues and organs, simultaneously. That ~~is~~was done by first amplifying the relevant fragments with the use of specific primers designed as to only specifically amplify those fragments of the bisulfite treated DNA that contain informative CpG positions. These amplicates ~~are~~were labelled with a fluorescent dye. A set of detection oligos, each designed as to specifically only hybridise with the amplified version of the bisulfite treated nucleic acid that ~~is~~was methylated as it is characteristic for a specific organ. The detection oligos contain a CG when said CpG position is methylated in a specific organ or tissue (or a TG where said CpG position is unmethylated in a specific organ or tissue). These oligos ~~are~~were fixed to a solid surface as to provide a chip. The labelled amplicates ~~are~~were hybridised with said chip and non hybridising amplicates ~~are~~were removed. The signal pattern on the chip ~~is~~was then translated in a methylation pattern, indicative of a specific organ.

The analysis of the patient's DNA methylation patterns, ~~lead~~s led to the conclusion that a significant portion of the DNA originated from colon.

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The physician therefore ~~initiates~~~~initiated~~ a second analysis on said bisulfite treated DNA. He ~~requires~~~~required~~ the patient's DNA to be tested a second time, this time specifically only with the colon marker EYA 4. A predominant signal ~~can~~~~could~~ be detected using the following EYA4-HeavyMethyl MethyLight assay. The methylation status ~~is~~~~was~~ determined with a HM MethyLight assay designed for the CpG island of the EYA4 colon marker gene and a control gene was assayed in parallel. The CpG island assay covers CpG sites in both the blocking oligos and the taqman® style probe, while the control gene does not.

Methods. The CpG island assay (methylation assay) ~~is~~~~was~~ performed using the following primers and probes:

Control gene : beta actin (Eads et al., 2001):

Primer: TGGTGATGGAGGAGGTTTAGTAAGT (SEQ ID No. 1);

Primer: AACCAATAAACCTACTCTCCCTTAA (SEQ ID No. 2); and

Probe: ACCACCACCAACACACAATAACAAACCA (SEQ ID No. 3)

EYA4 gene

Forward Primer: GGTGATTGTTTATTGTTATGGTTTG (SEQ ID No. 4)

Reverse Primer: CCCCTCAACCTAAAACTACAAC (SEQ ID No. 5)

Forward Blocker: GTTATGGTTTGTGATTTTGTGTGGG (SEQ ID No. 6)

Reverse Blocker: AAACCTACAACCACTCAAATCAACCCA (SEQ ID No. 7)

Probe: AAAATTACGACGACGCCACCCGAAA (SEQ ID No. 8).

The reactions ~~are~~~~were~~ each run in triplicate on the individual's DNA sample with the following assay conditions;

Reaction solution: (400 nM primers; 400 nM probe; 10µM both blockers; 3.5 mM magnesium

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chloride; 1x ABI Taqman buffer; 1 unit of ABI TaqGold polymerase; 200 μ M dNTPs; and 7 μ l of a solution containing 50 ng of DNA, in a final reaction volume of 20 μ l);

Cycling conditions: (95°C for 10 minutes); (95°C for 15 seconds, 64°C for 1 minute (2 cycles)); (95°C for 15 seconds, 62°C for 1 minute (2 cycles)); (95°C for 15 seconds, 60°C for 1 minute (2 cycles)); and (95°C for 15 seconds, 58°C for 1 minute, 60°C for 40 seconds (41 cycles)).

The amplification of said fragment ~~indicates~~~~indicated~~ the presence of a specific methylation pattern in said informative CpG positions (of EYA 4). From comparing the test result and the intensity of the fluorescent signal with a data set obtained from other samples it ~~can~~~~could~~ be concluded that a significant part of the DNA in the ~~patient's~~~~patients~~ sample originated from colon. This result ~~allows~~~~allowed~~ the physician to refer said patient to an expert in gastrointestinal diseases.

EXAMPLE 3

In another case the physician ~~follows~~~~was following~~ a different strategy. He ~~is~~~~was~~ first testing for the total amount of free floating DNA in said patient's serum, because this test is less cost intense and ~~is~~~~was~~ covered by the patient's insurance. The blood sample ~~is~~~~was~~ sent to a laboratory. After ~~separating~~~~having separated~~ the plasma from blood cells by centrifugation at 3000g for 20 min the DNA from the blood plasma ~~is~~~~was~~ extracted using the QIAamp Blood Kit (Qiagen, Hilden, Germany) using the blood and body fluid protocol referring to Wong et al. (1999), Cancer Res 59: 71-73 and Lo et al. (1998) Am. J. Genet. 62: 768-775. It ~~is~~~~was~~ determined that the level of total free floating nucleic acids in said serum sample ~~is~~~~was~~ 20 times higher than it usually is in samples from healthy donors, that are not suffering from cell proliferative diseases. The data ~~that were~~ establishing this "normal" value ~~are~~~~have been~~ obtained ~~from~~~~in~~ previous studies based on a high number of samples and ~~are~~~~were~~ approved by the regulatory agencies. These data ~~being~~~~had been~~ stored in their dataset.

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Knowing that his patient ~~has~~ had a level of free floating DNA in his serum that ~~is~~ was 20 times higher than the average ~~allows~~ allowed the physician to diagnose a high likelihood of his patient to suffer from a cell proliferative disease. With this diagnosis the insurance ~~is~~ was willing to pay for a more informative test as to further specify the kind of disease.

The physician now ~~requests~~ requested the methylation analysis of said DNA with the aim to determine where the free floating DNA in the serum of his patient originated from. Said DNA ~~is~~ was treated with sodium bisulfite as described above. The methylation pattern analysis ~~is~~ was carried out with the use of a number of informative CpG site containing marker nucleic acids and the collected datasets from other samples to compare the results with (as illustrated in figure 4). Said analysis ~~reveals~~ revealed that a significant portion of said free floating DNA ~~originates~~ originated from liver. At this point the physician ~~refers~~ referred the patient to an oncologist specified for liver tumours.

EXAMPLE 4

A research team is interested in identifying risks of developing lung specific diseases like for example lung cancer in a population, that has been exposed to specific environmental conditions. As these conditions only developed during ~~the~~ recent years, no data are available on an accumulated occurrence of cancer in said population yet. Therefore ~~the team is~~ they are employing an analysis of said ~~individual's~~ individuals bodily fluids as to whether they can find early signs of developing diseases. Sputum samples ~~are~~ have been collected from a high number of individuals.

Those sputum samples ~~are~~ were analysed as follows: Sputum samples ~~are~~ were spun at 3000 x g for 5 min and washed twice with phosphate-buffered saline. Cell pellets ~~are~~ were digested with 1% SDS/proteinase K, and DNA ~~is~~ was extracted and purified using Qiagen columns (Qiaamp Blood Kit, Qiagen, Basel, Switzerland) according to the "blood and body fluid protocol". The DNA obtained ~~is~~ was subjected to a sodium bisulfite treatment as it has been described in Olek A, Oswald J, Walter J. (1996) A modified and improved method for bisulphite based cytosine

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methylation analysis. Nucleic Acids Res. 24: 5064-6. An aliquot of this bisulfite treated DNA ~~is~~was used for methylation analysis. As the study ~~is~~was designed to only look for lung diseases, the analysis ~~is~~was restricted to the use of informative CpG sites that are specifically methylated in lung cells, but unmethylated in other cells that might potentially occur in a sputum sample. The methylation analysis ~~is~~was based on sensitive detection assays. First results ~~are~~were obtained with the use of a HM assay, as it is described in here. Primers ~~are~~were designed to amplify a fragment that contains seven different CpG sites that are all methylated only in DNA from lung cells. Blocking oligos ~~are~~were designed that hybridised to two of those sites in the bisulfite treated DNA, only when said CpG sites ~~are~~were unmethylated prior to bisulfite treatment. A pair of Lightcycler probes ~~is~~was designed as to only bind to the amplified fragment of the bisulfite treated DNA when two different informative CpG sites ~~are~~were methylated. That way, the presence ~~is~~was indicated by the generation of a fluorescent signal and the amount of said lung derived DNA in the total amount of DNA ~~is~~was quantified by the number of cycles required to generate a detectable signal in comparison to signals generated by standardised amounts of control DNA.

The primary test results ~~are~~have been confirmed with the use of MSP primers in combination with the use of Taqman probes. MSP primers ~~are~~were designed to specifically bind to the bisulfite treated sequence containing two and three of those CpG sites that ~~are~~were methylated in lung cells, but not in other cells. The Taqman probe ~~is~~was designed to bind to the other two CpG sites in said amplified product only when those ~~are~~were unmodified after treatment with bisulfite (methylated cytosines prior to treatment). Therefore the presence of an amplification product, indicated by the fluorescent signal of the Taqman probe ~~confirm~~confirmed the primary results.

As the majority of the individuals ~~do~~did not show free floating DNA in their sputum samples that ~~exhibit~~exhibited methylation pattern characteristic for lung, it ~~is~~was concluded that they ~~do~~did not contain lung derived DNA in their sputum samples. It ~~is~~was concluded that said population did not develop lung specific cell proliferative diseases and as such there ~~is~~was no reason to believe that said environmental conditions ~~are~~were adding to the risk of developing a neoplastic or

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inflammatory diseases like lung cancer or lung inflammation.